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# New insights into the Fanconi anemia pathway from an isogenic *FancG* hamster CHO mutant

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**Abstract**

The Fanconi anemia (FA) proteins overlap with those of homologous recombination through FANCD1/BRCA2, but the biochemical functions of other FA proteins are largely unknown. By constructing and characterizing a null *fancg* mutant (KO40) of hamster CHO cells, we show that FancG protects cells against a broad spectrum of genotoxic agents. KO40 is consistently hypersensitive to both alkylating agents that produce monoadducts and those that produce interstrand crosslinks. KO40 cells were no more sensitive to mitomycin C (3×) and diepoxybutane (2×) than to 6-thioguanine (5×), ethylnitrosourea (3×), or methyl methanesulfonate (MMS) (3×). These results contrast with the pattern of selective sensitivity to DNA crosslinking agents seen historically with cell lines from FA patients. The hypersensitivity of KO40 to MMS was not associated with a higher level of initial DNA single-strand breaks; nor was there a defect in removing MNU-induced methyl groups from DNA. Both control and MMS-treated synchronized G1-phase KO40 cells progressed through S phase at a normal rate but showed a lengthening of G2 phase compared with wild type. MMS-treated and untreated early S-phase KO40 cells had increased levels of Rad51 foci compared with wild type. Asynchronous KO40 treated with ionizing radiation (IR) exhibited a normal Rad51 focus response, consistent with KO40 having only slight sensitivity to killing by IR. The plating efficiency and doubling time of KO40 cells were nearly normal, and they showed no increase in spontaneous chromosomal aberrations or sister chromatid exchanges. Collectively, our results do not support a role for FancG during DNA replication that deals specifically with processing DNA crosslinks. Nor do they suggest that the main function of the FA protein "pathway" is to promote efficient homologous recombination. We propose that the primary function of FA proteins is to maintain chromosomal continuity by stabilizing replication forks that encounter nicks, gaps, or replication-blocking lesions.


*Keywords:* FANCG; Fanconi anemia; Replication fork; Rad51 foci; Homologous recombination

## 1. Introduction

Fanconi anemia (FA) is a rare autosomal recessive disorder characterized by progressive bone marrow failure and cancer susceptibility, especially for acute myelogenous leukemia [1]. FA is genetically heterogeneous, consisting of at least eight complementation groups for which genes have been identified: *FANCA*, *C*, *D1*, *D2*, *E*, *F*, *G*, and *FANCL/PHF9* [2,3]. The genes for groups B, I, and J are unidentified [4]. The role of the FA protein "pathway" in recovery of cells from DNA damaging agents is under intense investigation. FA A/C/E/F/G/L proteins interact to form a nuclear complex [3,5–10]. The integrity of this complex is essential for the monoubiquitination of FANCD2 following mitomycin C (MMC) or ionizing radiation (IR) treatment; this modifica-

*Abbreviations:* MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; MNU, methylnitrosourea; ENU, ethylnitrosourea; CNU, chloroethylnitrosourea; IR, ionizing radiation; MMC, mitomycin C; HR, homologous recombination; HRR, homologous recombinational repair; ROS, reactive oxygen species; SCE, sister chromatid exchange; DSB, double-strand break; $S^6G$, 6-thioguanine

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tion of FANCD2 appears essential for normal resitance to genotoxic agents. Interestingly, FANCD1, D2, and L are the only FA proteins evolutionarily conserved in non-vertebrates [3,11,12].

Recently, the *FANCD1* gene was found to be identical with *BRCA2* [13], a participant in the formation of Rad51 nucleoprotein filaments during homologous recombinational repair (HRR) [14]. FA-D1 and FA-J mutant cells are able to monoubiquitinate FANCD2 at Lys561 [15], whereas cells in the other groups cannot ubiquitinate FANCD2 during S phase of the cell cycle [4,16]. The finding that FANCD1 is a bonafide homologous recombination protein suggests that the main function of the FA proteins may be to promote HRR [2]. Indeed, monoubiquitinated FANCD2 colocalizes with BRCA1 and Rad51 in nuclear foci [15,16].

The phenotype of FA cells includes increased chromosomal breakage/exchange, apoptosis, and reactive oxygen species (ROS), as well as prolongation of the G2 phase (for reviews see [2,17–19]). One possible mechanism is that elevated ROS leads to genotoxic stress. Many studies have suggested defects in oxygen metabolism in FA cells (reviewed in [20]); at least FANCC [21,22] and FANCG [23] appear to have important roles in redox metabolism.

Historically, FA has often been viewed as a DNA repair deficiency disorder, largely because FA cells are consistently hypersensitive to DNA cross-linking agents and have increased chromosome fragility [24,25]. The evidence for defects in DNA repair has been inconsistent and contradictory [26,27]. The lack of homology between many FA proteins and DNA repair proteins in lower organisms argues against the involvement of FA proteins in the enzymology of DNA repair.

Previously we cloned the human *XRCC9* gene by phenotypic correction of the MMC sensitivity of a mutagen-derived CHO mutant (UV40) [28]. Despite the UV sensitivity of UV40, *XRCC9* proved to be identical to *FANCG* cloned from FA-G lymphoblasts [29]. This convergence was unexpected because UV sensitivity is generally not associated with FA cells. In order to have a better-defined, isogenic CHO *fancg* system, we produced a knockout event by gene targeting. Our studies of this new mutant show a broad spectrum of genotoxin sensitivity that is inconsistent with a specific deficiency in processing DNA interstrand crosslinks. The increased sensitivity to methylating damage in *fancg* mutant cells is not due to increased initial damage. The phenotype of *fancg* cells suggests a more general role for FA proteins than participating in HRR when DNA replication encounters lesions.

## 2. Materials and methods

### 2.1. Cell culture and centrifugal elutriation

CHO cells were grown in monolayer or suspension culture in αMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were counted and analyzed on a Coulter® Multisizer II during subculturing. Cell doubling time was determined for suspension cultures diluted twice weekly by averaging 20 consecutive measurements. In synchronization experiments, $2 \times 10^8$ cells were resuspended in elutriation buffer (80% PBS and 20% culture medium). The buffer and cells were kept on ice, and the centrifuge was set at 8 °C. Centrifugal elutriation was conducted in elutriation buffer on a J6-M1 centrifuge (Beckman Coulter) at 2800 rpm with an initial flow rate of 19–20 ml/min and ∼20 fractions were collected as the flow rate was incremented by 1 ml/min.

### 2.2. FancG targeting vector construction

Gene-targeting plasmid pUC-FancG.TV was derived from pUC19. The multiple cloning region of pUC19 was replaced with a new cloning region containing a short section of the neomycin gene (*neo*) beginning at the ATG start codon and ending at the *Eag*I restriction site by inserting an 82-mer duplex oligonucleotide between *Eco*RI and *Hin*dIII. The remaining *neo* sequences from *Eag*I to *Sal*I were cloned from IRES-Neo-pA (a gift from John Sedivy, Brown University) to create plasmid pUC-neo. Two *FancG* recombinant PCR fragments were amplified from a CHO *FancG* genomic BAC clone (BAC 174) and inserted into the two cloning sites of pUC-neo. The upstream *FancG* PCR fragment from intron 1 to exon 3 was 1419 bp and cloned in frame with *neo*, and the downstream *FancG* PCR fragment from exon 7 to exon 14 was 3987 bp. The thymidine kinase gene (*tk*) was isolated from pSSC-9 [30] and blunt-end ligated into the final vector to create pUC-FancG.TV. Plasmid pUC-FancG.con was created as a positive control for gene targeting by defining PCR reactions that would identify *FancG* knockout clones. pUC-FancG.con contains an additional 655 nt 5′ of the upstream *FancG* fragment inserted into pUC-FancG.TV. PCR reactions were performed on 0.5 μg genomic DNA using High-Fidelity Platinum® Taq DNA Polymerase (Invitrogen). PCR primer sequences are available upon request.

### 2.3. Gene targeting and DNA transfection

For gene targeting, $3 \times 10^7$ cells were rinsed with and resuspended in 1 ml cold electroporation buffer (20 mM HEPES (pH 7), 137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$, 6 mM glucose), mixed with 10 μg linearized pUC-FancG.TV DNA, electroporated at 250 V/1600 μF, incubated for 5 min on ice, and plated in T150 flasks for 24 h to allow for *neo* expression. Cells were plated into 10 cm dishes at $\sim 2 \times 10^6$ cells per dish in 20 ml medium containing 1.7 mg/ml G418 (Gibco Invitrogen) and incubated for 5 days, after which the medium was replaced with fresh medium (supplemented with 10% dialyzed serum) containing 0.1 μM 2′-fluoro-2′-deoxy-1-beta-D-arabinofuranosyl-5-iodouracil (FIAU); cells were incubated for an additional 5 days. Each dish contained a pool of ∼150 drug-resistant colonies, which were harvested for freezing and DNA isolation (QIAamp® DNA Blood Mini Kit, Qiagen Inc). The

frequency of G418 resistant colonies averaged $3.6 \times 10^{-4}$, and the FIAU enrichment was approximately five-fold. Gene-targeted clones were identified and isolated through two rounds of screening, first into sub-pools of 20 clones and then individual clones.

### 2.4. Mutagen sensitivity

Mutagen sensitivity was determined by colony formation in 10 cm dishes. When most colonies were clearly visible by eye, dishes were rinsed with saline, fixed with 95% ethanol and stained with Gram Crystal Violet (Becton Dickinson). Exposure to genotoxic agents was as follows: UV radiation [31]; $^{137}$Cs γ-irradiation, in 15 ml polypropylene tubes kept on ice; for MMS, MNU, ENU, CNU, and MMC, $1 \times 10^5$ cells/ml in 10 ml tube suspension cultures were exposed at 37 °C for 60 min, chilled on ice, centrifuged, resuspended in fresh medium; for hydroxyurea, paraquat, and camptothecin, cells were exposed on dishes for 24 h; for $H_2O_2$, cells were rinsed and exposed in PBS for 30 min at room temperature; for 6-thioguanine ($S^6G$) and thymidine cells were treated continuously. Treatment with EMS, diepoxybutane, chlorambucil, bleomycin, streptonigrin, epoxybutane, and etoposide was continuous as described [32]. The assessment of $S^6G$ incorporation and toxicity was done using 10% dialyzed fetal bovine serum, 10 μg/ml mycophenolic acid, 10 μg/ml guanine, and varying concentrations of $S^6G$.

### 2.5. Rad51 immunofluorescence

Ten millilitres of cell suspension at $1 \times 10^5$ cells/ml was treated with 75 μg/ml MMS for 1 h, rinsed once with medium, and resuspended in 10 ml fresh medium. Cells were incubated for 4 h and then centrifuged onto glass slides at 2000 rpm for 5 min using a Cytospin® 4 cytocentrifuge (Thermo Shandon). Cells were fixed in 2% paraformaldehyde for 15 min, permeabilized in cold 0.2% Triton X-100 for 5 min, and blocked in 1% BSA for 1 h. The slides were incubated with anti-Rad51 antibody (clone H-92, Santa Cruz Biotechnology) at 4 °C overnight (1:1000 dilution in 1% BSA), and Alexa Fluor® 488 goat anti-rabbit secondary antibody (A-11008; Molecular Probes) at room temperature for 1 h. Glass slides were mounted using Vectashield mounting medium with DAPI (H-1200; Vector Laboratories). Fluorescence images were captured on Quips PathVysion using an Axiophot II fluorescence microscope, and Rad51 foci were counted visually.

### 2.6. DNA methylation damage and repair

Cells were exposed to *N*-[$^3$H]methyl-*N*-nitrosourea at 46–93 ng/ml (Amersham Pharmacia Biotech) for 30 min at 37 °C in suspension culture at $2 \times 10^6$ cells/ml in 10 ml medium made acidic by sparging with 100% $CO_2$. Cells were washed twice with medium, resuspended in 10 ml fresh medium, and incubated at 37 °C to allow for repair. Genomic DNA was isolated from 2 ml volumes at the times noted using DNeasy tissue kit (Qiagen) with inclusion of an RNase A digestion step. DNA methylation was determined by disintegration per min (dpm) per μg DNA.

Alkaline single-cell gel electrophoresis was used to measure DNA damage [33]. Briefly, slides with cells in agarose were prepared, placed in lysis buffer composed of 1% Triton X-100, 10% DMSO, 89% stock lysing solution (2.8 M NaCl, 0.1 M $Na_2EDTA$, 0.01 M Trizma Base) overnight at 4 °C; after rinsing in 0.4 M Tris, pH 7.5 and immersing in 300 mM NaOH, 1 mM EDTA, pH >13.0 for 60 min, they were electrophoresed at 28 V at 300 mA for 25 min. Slides were stained with ethidium bromide (2 μg/ml) and images of 100 cells analyzed to determine comet distributed moment [34] using Komet4.0©: Image Analysis and Data Capture (Kinetic Imaging Ltd., Merseyside, England).

### 2.7. Cell cycle progression

Cells were collected by centrifugal elutriation as synchronous populations in late G1 and were treated (or not) with 1.8 mM MMS for 8 min at 37 °C (see above). Progression through the cell cycle was determined by analyzing DNA-content distributions at 2 h intervals. The cells were treated with 10 μg/ml BrdUrd for the final 20 min before sample collection, fixed with 70% EtOH, and stained with FITC-conjugated anti-BrdUrd antibody (BD Biosciences) and propidium iodine. Fluorescence measurements of each sample were made on a FACscan (Becton Dickinson) and the data analyzed using Cell Quest software.

## 3. Results

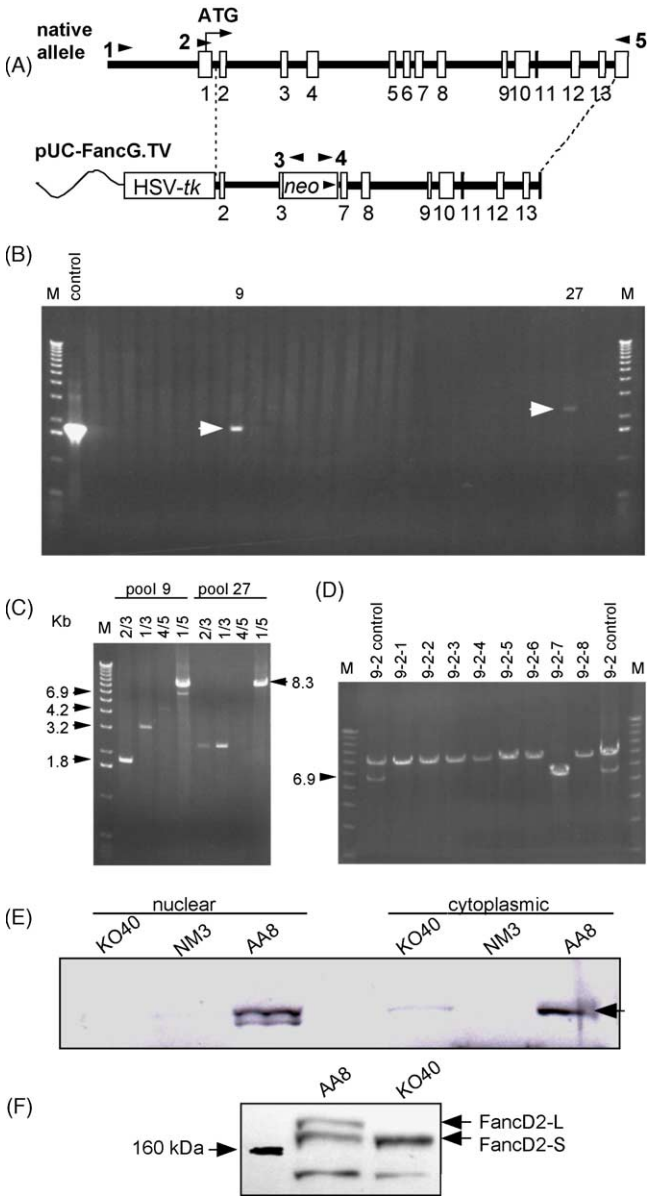
### 3.1. Fancg knockout cells occur at low frequency

Although CHO AA8 cells are hemizygous at the *FancG* locus [35], gene targeting occurs infrequently relative to random integration, requiring a screening assay to detect a knockout event in pools of transformant clones. We designed a targeting vector that contains a promoterless *neo* gene fused in-frame with *FancG* exon 3 for positive selection in G418 and the *HSV-tk* gene for negative selection in FIAU (Fig. 1A). Gene targeting through HR replaces *FancG* exons 4–6 with *neo*, reducing the genomic sequence by 1.4 kb.

Transformants were screened by PCR in pools of ∼100–200 clones in three steps. First, we tested for HR of the left arm of the targeting vector by PCR amplification from *neo* to upstream *FancG* sequence (Fig. 1B). Second, pools were tested by PCR across the right arm from *neo* to downstream *FancG* sequence, and finally across the entire targeted (shortened) gene (Fig. 1C). A single pool of clones containing a knockout event was processed through two successive rounds of PCR screening, first in sub-pools of 20 clones (not shown) and then as single clones (Fig. 1D). Three targeted subclones (KO40, KO50 and KO90), which are likely siblings, were isolated from >12,000 clones, giv-

ing a targeting frequency of $\sim 10^{-4}$. Clone KO40, used in most work described here, was confirmed by Western blot to be a null mutant (Fig. 1E); we refer to these cells as *fancg* cells. FA-G mutations are associated with loss of monoubiquitination of FANCD2 [15]. A Western blot of FancD2 confirmed this deficiency in exponentially growing KO40 cells (Fig. 1F).

Clone KO40 was complemented with CHO *FancG* BAC-clone DNA to produce a pool of six independent transformants (40BP6) that was resistant to MMC and expressed FancG at the same level as in AA8 cells (data not shown). KO40 cells have a slightly longer doubling time compared to AA8 (KO40 = 13.9 ± 0.6 h [SEM]; AA8 = 13.0 ± 0.6 h; and 40BP6 = 13. 7 ± 1.0 h) and demonstrate nearly normal plating efficiency (84% versus 90% for AA8 and 40BP6).




### 3.2. Fancg cells exhibit diverse mutagen sensitivity

KO40 cells exhibited increased sensitivity to a variety of agents (Fig. 2; see numerical summary in legend). As expected, KO40 cells are sensitive by two- to three-fold to the DNA interstrand cross-linking agents diepoxybutane, mitomycin C, chloroethylnitrosourea, and chlorambucil. However, the mutant's sensitivity to the closely related compounds ethylnitrosourea (noncrosslinker) and chloroethylnitrosourea (crosslinker) was similar, illustrating that KO40 cells are not preferentially sensitive to crosslinking agents. Furthermore, KO40 cells have four-fold sensitivity to both methyl methanesulfonate (MMS) and methylnitrosourea (MNU). MMS and MNU preferentially methylate DNA on nitrogen and oxygen atoms, respectively. Surprisingly, $S^6G$ sensitivity increased five-fold. KO40 cells are mildly sensitive to UV-C radiation (1.5-fold), but only slightly sensitive to γ-irradiation (1.2-fold), camptothecin, and hydroxyurea. We ruled out the possibility that the $S^6G$ sensitivity might be due to more efficient uptake and incorporation of guanine by KO40 versus AA8 cells by using conditions that required the cells to grow on exogenous guanine (data not shown).

### 3.3. Fancg cells exhibit wild-type levels of binding and removal of DNA methyl groups

The sensitivity of KO40 cells to methylating agents could result from either elevated DNA damage or a defective response in removing, or coping with, base damage. The possibility of more initial DNA damage in KO40 cells could con-

Fig. 1. *FancG* knockout in CHO AA8 cells. (A) Physical map of hamster *FancG* and targeting vector pUC-FancG.TV. Open boxes represent coding regions, thick lines represent *FancG* intronic sequences, and the thin line represents plasmid sequences. *HSV-tk* and *neo* are labeled and *FancG* exons are numbered. Homologous sequences between FancG and pUC-FancG.TV are indicated by dotted lines. Arrowheads are PCR primer binding sites. Homologous recombination deletes *FancG* exons 4–6 and inserts *neo* in frame at *FancG* exon 3, allowing for PCR amplification between corresponding *FancG* and *neo* PCR primers. (B) PCR screening pools of AA8 transformants for the presence of *FancG* knockout cells. PCR amplification was from primers 2 and 3 (targeted amplification product for PCR 2/3 is 1845 bp). Plasmid pUC-FancG.con served as a positive control. The arrowheads mark the pools containing potentially targeted cells. (C) First round PCR positive pools 9 and 27 were examined with additional PCR primers as listed. (Targeted amplification products: PCR 1/3 = 3241 bp; PCR 4/5 = 4230 bp; and PCR 1/5 = 6860 bp. Wild-type amplification product for PCR 1/5 = 8270 bp.) The expected PCR product sizes for knockout cells are shown on the left side of the gel, and the WT PCR product size is shown on the right side. (D) *FancG* knockout subclones were identified by PCR screening of single-cell clonal isolates using primers 1 and 5. Control sample 9–2 is a 20-cell sub-pool of pool 9, positive for gene targeting, from which the subclones were derived. Note that subclone 9-2-7 contains no WT PCR band. Markers are 1 kb DNA ladder (Invitrogen, Carlsbad, CA). (E) Western blot showing loss of FancG in KO40, and NM3 cells, which have a frame-shift mutation [35]. (F) FancD2 Western blot of nuclear extracts of wild type and *fancg* mutant cells. FancD2-L and -S (long and short) are the ubiquitinated and non-ubiquitinated forms of FancD2, respectively.

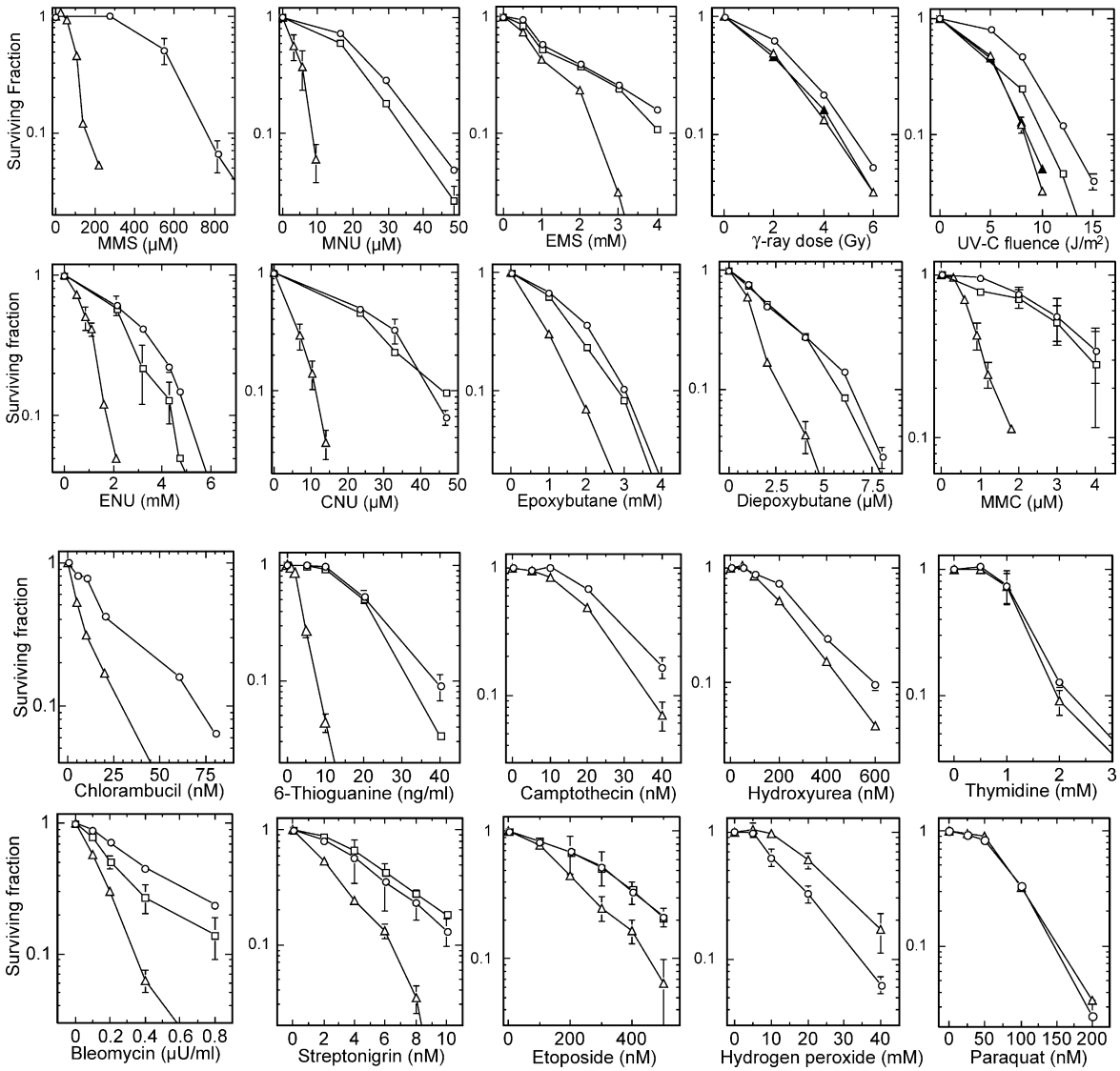



Fig. 2. Colony formation survival curves in response to DNA damaging agents and DNA replication inhibitors. The increased sensitivity of KO40/90 cells, based on $D_{37}$ values is in the following rank order: 6-thioguanine ($S^6G$) (5×), methyl methanesulfonate (4×), methyl-nitrosourea (4×), mitomycin C (3×), ethyl-nitrosourea (3×), chloroethyl-nitrosourea (3×), diepoxybutane (2×), chlorambucil (2×), bleomycin (2×), streptonigrin (2×), epoxybutane (1.9×), ethyl methanesulfonate (1.6×), UV-C radiation (1.5×), etoposide (1.6×), camptothecin (1.2×), hydroxyurea (1.2×), γ-rays (1.2×), paraquat (1.0×), thymidine (1.0×), $H_2O_2$ (0.8×). All agents except MMS, γ-rays, and epoxybutane were tested in two or three experiments. Error bars represent standard errors. Symbols: AA8 (◯); KO40 (△); KO90 (▲); and 40BP6 (□). Note that KO40 and KO90 are sibling subclones (γ-ray and UV-C panels).

ceivably arise from an altered redox status [20]. We tested whether KO40 cells had increased levels of DNA damage, as reflected by single-strand breaks. No difference in spontaneous DNA damage was seen using the "comet" assay (Fig. 3A). For treatment with MMS, we used a short-duration exposure to minimize the extent of repair during the exposure period and thereby provide an estimate of the "initial" damage reflected in increased breaks. In this instance, breaks arise as repair intermediates during base excision repair. Again, no difference was seen between KO40 and AA8 cells in the level of DNA breaks immediately after an 8 min exposure to MMS (Fig. 3A). These results of equal damage were confirmed using *N*-[$^3$H]methyl-*N*-nitrosourea ([$^3$H]MNU) and measuring specific activity in DNA (Fig. 3B). KO40 cells experience the same amount of DNA methylation as AA8 cells. Moreover, the rates of reduction in specific activity of the DNA during 24 h post-treatment incubation were the same (Fig. 3C). We conclude that *fancg* cells have normal removal of methylation damage and that their sensitivity is likely caused by defective replication, resulting in excess double-strand breaks (DSBs).

### 3.4. *Fancg cells do not show elevated reactive oxygen species*

Substantial evidence suggests a defect in oxygen metabolism in FA cells, as recently reviewed [20]. FANCG interacts with cytochrome P4502E1 (CYP2E1), and *fancg*

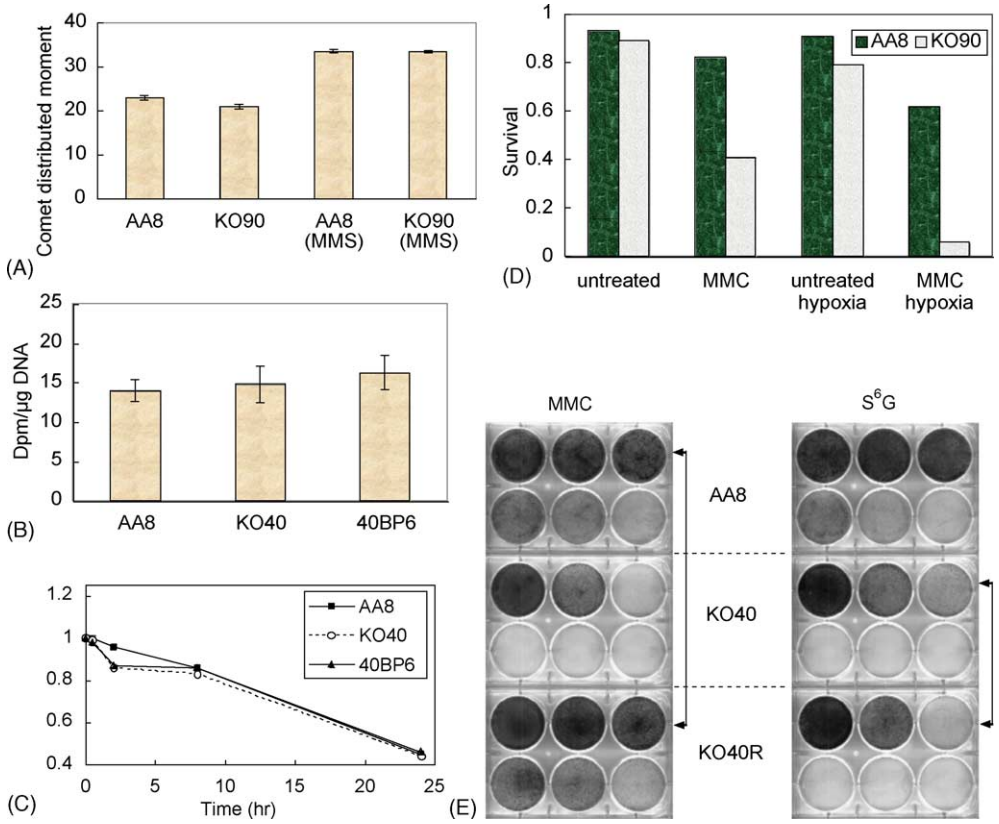



Fig. 3. Measurement of single-strand breaks and methylation adducts. (A) DNA damage measured by alkaline single-cell gel electrophoresis (comet) with and without exposure to 1.1 mM MMS for 8 min, which resulted in 76% survival of AA8 cells versus 16% survival of KO40 cells. Values are the mean of 100 data points and error bars are standard errors. (B) DNA damage measured as specific activity of genomic DNA from cells exposed to 46–93 ng/ml [$^3$H]MNU for 30–60 min. Values represent three experiments and error bars are standard errors. 40BP6 is a pool of six transfectants of KO40 that express the hamster *FancG* gene. (C) Rate of repair measured as specific activity of genomic DNA as a function of time following a 30 min exposure to 93 ng/ml [$^3$H]MNU. The data were normalized to the amount of damage detected at time 0 and corrected for dilution of signal due to cell division. The graph represents one experiment. (D) MMC sensitivity under hypoxic conditions. KO90 and AA8 cells were incubated under standard (21% $O_2$) or hypoxic (<100 ppm $O_2$) conditions for 1 h at 37 °C in the presence or absence of 1.5 μM MMC. Cellular sensitivity was determined by colony formation. Results are the average of three dishes (one experiment). (E) Phenotypic suppression of MMC sensitivity in MMC-resistant derivatives of KO40 cells. One mitomycin resistant clone (KO40R) derived from KO40 was compared with AA8 and KO40 cultures for sensitivity to killing by MMC and $S^6G$ in a differential cytotoxicity assay [77] using graded doses of each agent. (From left to right and top to bottom rows, the MMC concentrations were 0, 29, 51, 93167 and 300 nM, and the $S^6G$ concentrations were 0, 2.5, 5.0, 10, 20 and 40 ng/ml). Twenty thousand cells per well were seeded and exposed continuously for 4 days (AA8) or 5 days (KO40). The cells were then fixed with ethanol and stained; the amount of cell growth is visualized by the intensity of staining.

null lymphoblasts show increased CYP2E1, suggesting that FANCG contributes to down regulating this protein [23]. CYP2E1 is a major producer of ROS, including superoxide and hydrogen peroxide [36,37]. The half-life of FANCG is reduced ∼10-fold in mutant *FANCA* cells, suggesting FANCA could reduce ROS indirectly by stabilizing FANCG [38]. We measured ROS by fluorescence of the dye CM-H2DCFDA (Molecular Probes), which was used to detect increased ROS in chromosomally unstable CHO cells [39]. We did not detect any difference in fluorescence among AA8, KO40, and 40BP6 cells (data not shown).

We also tested the hypothesis that the sensitivity of FA cells to MMC is caused by the increased production of reactive oxygen species (ROS) at ambient oxygen levels [40]. We measured the MMC sensitivity of cells treated for 1 h under hypoxic conditions with 95% $N_2$/5% $CO_2$ ($O_2$ level: <100 ppm) [41]. Hypoxic conditions potentiated the MMC toxicity in both cell lines, and actually increased the relative MMC sensitivity of *fancg* cells (Fig. 3D). Thus, the hypothesis of Clarke does not account for the MMC sensitivity of *fancg* CHO cells [40].

### 3.5. *Fancg cells have an elevated rate of conversion to MMC resistance*

MMC-resistant cells are present in *fancg* cultures. Under selection at 150 nM MMC, colonies arise at high frequency ($\sim 10^{-4}$). Several of these phenotypic revertant clones were isolated and examined for the level of MMC and $S^6G$ resistance. As shown in Fig. 3E for a clone designated KO40R, only MMC resistance is increased relative to KO40. KO40R cells have the same $S^6G$ sensitivity as KO40 (see comparison indicated by right arrows in Fig. 3E). KO40R cells are as resistant to MMC as the AA8 parental cells (left arrows in

Fig. 3E). These results suggest that some function affecting MMC metabolism is altered in these phenotypic revertants. In contrast, the parental AA8 cells do not show such phenotypic instability.

### 3.6. *Fancg* cells with methylated DNA are not delayed during S phase

We wished to determine whether methylation sensitivity of *fancg* cells was associated with a defect in progression through S or G2/M phases. Centrifugal elutriation was used to obtain highly synchronous cell fractions of exponentially growing cultures to study cell cycle progression. The starting fractions were collected as predominantly late G1 cells (∼10% cells in S phase incorporating BrdUrd). The tight synchrony of both AA8 and KO40 cells immediately after isolation is illustrated in Fig. 4A. A synchronous wave of cells entered S phase, peaked at about 6 h, and then declined up to 12 h (Fig. 4B). *Fancg* cells entered and progressed through S phase slightly behind AA8 cells (∼1 h delay), which suggests a lower rate of synthesis at the beginning of S, or alternatively might reflect different positions in G1 at $t = 0$. Replicate cultures treated with MMS (1.8 mM) for 8 min were not delayed in progression through S phase in comparison with untreated cells although only 20% of the *fancg* cells survived in terms of colony forming ability. Analysis of cell entry and exit of the G2/M phase showed a slightly prolonged G2 phase, by 1–2 h, for *fancg* cells in the absence of MMS treatment (Fig. 4C). MMS damage resulted in a longer G2-phase for both cell types, but the difference between them remained similar to the untreated samples.

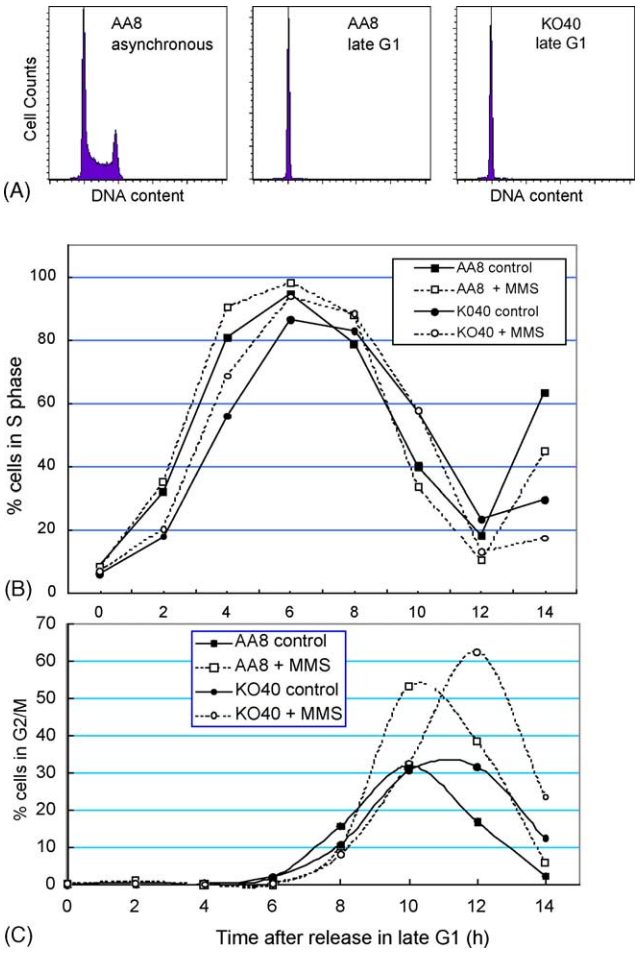



Fig. 4. Cell cycle progression of *fancg* and control cell lines. (A) Synchronous fractions were collected from exponentially growing cultures by separating cells in late G1 phase (>90% purity). (B) Synchronous cells as shown in Panel A were allowed to progress through the cycle with, or without, exposure to 1.8 mM MMS for 8 min, which resulted in 20% survival of KO40 cells and 80% of AA8 cells, as measured by colony forming ability. To measure the percentage of cells that were synthesizing DNA, samples were pulse-labeled with BrdUrd at the time point shown and stained with BrdUrd-specific antibody for analysis by flow cytometry. The data points are the average of two independent experiments. (C) The data from the experiments shown in Panel B are shown for the percentage of cells in G2/M at various times. The curves were smoothed mathematically for ease of viewing. The area under each curve approximates the average length of G2/M for each cell type.

### 3.7. *S-phase fancg cells have elevated levels of Rad51 nuclear foci*

Nuclear foci of Rad51 protein occur spontaneously at low frequency during S-phase and increase in number and intensity in response to certain genotoxic agents such as IR, as recently reviewed in detail [42]. Rad51 focus formation is thought to reflect Rad51 concentrated at sites of HRR. As the Fanconi anemia pathway is linked to HRR through the BRCA1/2 proteins [2,43] and because *brca2* mutant cells have reduced levels of Rad51 foci, we examined Rad51 focus formation. Asynchronous KO40 cells exhibited a normal level of both spontaneous and γ-ray-induced Rad51 foci (Fig. 5A–C). These results suggest KO40 can initiate a normal HRR response following γ-radiation. This result is consistent with the nearly normal IR sensitivity of KO40 cells (Fig. 2). We note that there are conflicting results between two laboratories concerning whether diploid human FA-G fibroblasts have decreased IR-induced Rad51 foci [44,45].

To examine Rad51 foci associated specifically with DNA replication, we used cells synchronized in early S-phase. We saw an average increase of 2.2-fold in spontaneous Rad51 foci in KO40 versus AA8 S-phase cells (Fig. 5D). Notably, the level of Rad51 protein examined by Western blotting was the same in AA8 and KO40 cells (data not shown). Our failure to see increased Rad51 foci in asynchronous, untreated *fancg* cells may be explained by insufficient assay sensitivity with heterogeneous populations. We also observed an appreciable increase (approximately two-fold) in foci in S-phase KO40 relative to AA8 after exposure to MMS (Fig. 5E). Since KO40 cells are three-fold more sensitive to killing by MMS, these data suggest that they initiate more HRR repair events, which are manifest as Rad51 foci, because they experience more DSBs than AA8 cells. An alternative interpretation is

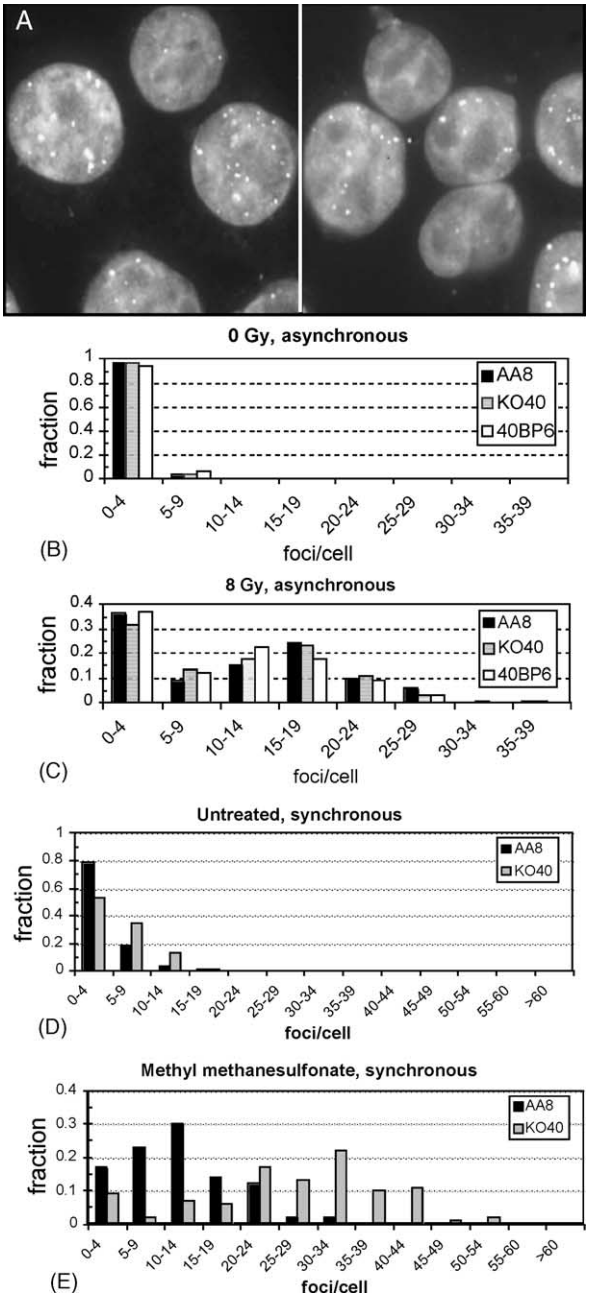



Fig. 5. Rad51 foci formation following γ-irradiation or MMS exposure. (A) Cells were exposed to 8 Gy of $^{137}$Cs and incubated at 37 °C for 4 h. Foci were counted visually under a Zeiss Axiophot upright microscope. (Left panel) AA8 cells; (right panel) KO40 cells. The results in Panels B–F represent counts from a minimum of 100 nuclei per sample. (B) and (C) Distribution of foci per cell in asynchronous unirradiated and irradiated cultures. (D) Distribution of foci in untreated S phase cells from one of two experiments. (E) For chemical treatment, early S-phase cells were isolated by centrifugal elutriation and exposed to 680 μM MMS for 1 h at 37 °C, washed, and incubated at 37 °C for 4 h before fixation. One of two experiments is shown.

that foci form at a normal rate in *fancg* cells but are more persistent because of a block in HRR downstream of Rad51 focus formation. However, this possibility seems unlikely since *fancg* cells show normal levels of IR-induced foci.

### *3.8. Spontaneous chromosomal breakage and sister-chromatid exchange are normal in fancg cells*

Since increased chromosomal breakage and exchange is a prominent feature of FA, we measured spontaneous chromosomal aberrations in KO40 cells versus the two control lines. Based on a sample size of 800–1000 cells scored by two individuals, we did not see a consistent increase in aberrations in *fancg* cells (Table 1). Although there was a slight elevation in the first scoring, this increase was not reproducible. A normal level of aberrations is consistent with the nearly normal plating efficiency of KO40 cells. Although this result was somewhat surprising, it is worthy noting that an analysis of chromosomal aberrations in FA-G lymphoblasts (line EUFA143) versus their gene-complemented control cells also did not reveal any difference (unpublished data).

We sought evidence for enhanced chromosomal breaks at the first metaphase in the 14 h samples of the cell synchrony experiments shown in Fig. 4 (Table 2). Surprisingly, there was no difference in aberration frequency between MMS-treated AA8 (6% abnormal metaphases; 80% survival) and KO40 (5% abnormal; 20% survival). However, this result does not exclude the presence of excess DSBs in *fancg* metaphase chromosomes that may be deleterious in the next cell cycle. Studies using γ-H2AX as a marker for DSBs clearly show that DSBs are often present in metaphase chromosomes at sites where chromosomal breaks are not cytologically visible [46,47]. Thus, we infer that a clear distinction must be made between DSBs whose ends remain in close proximity versus chromosomal breaks, which reflect major spatial dislocation of ends.

Sister-chromatid exchange (SCE) is one measure of homologous recombination (requiring crossing over) and is elevated in certain genome instability disorders such as Bloom syndrome. We found that the frequency of SCE is normal in *fancg* cells: 8.4 per cell (251 exchanges in 30 cells) for AA8 and 8.3 for KO40 cells (248/30). These results agree with the historical observation that FA cells generally do not show altered SCE frequencies [48].

## 4. Discussion

### *4.1. Isogenic fancg knockout cells differ from the UV40 and NM3 mutants*

We constructed a knockout mutant of *FancG* in the widely used quasi-diploid CHO line AA8 [49], which grows in both suspension and monolayer culture. One other knockout mutant was reported for a DNA damage-response gene in CHO cells, namely *ERCC1* [50]. The targeting efficiency for *FancG* was surprisingly >10-fold lower than that seen for *ERCC1*, despite the larger regions of homology in our vector. As an isogenic mutant, KO40 has major advantages over the CHO mutants UV40 and NM3, which belong to the same complementation group [32] and were produced by potent chemical

Table 1
Spontaneous chromosomal aberrations *fancg* cells

| Cell line | Cells scored | Abnormal cells[a] | Ctd[b] gaps | Ctd breaks | Ctd exchanges | Other | Percentage abnormal cells |
|---|---|---|---|---|---|---|---|
| First scorer | | | | | | | |
| AA8 | 600 | 12 | 13 | 13 | 0 | 1 | 2 |
| KO40 | 600 | 27 | 29 | 26 | 0 | | 4.5 |
| 40BP6 | 400 | 12 | 19 | 11 | 1 | 0 | 3 |
| Second scorer | | | | | | | |
| AA8 | 400 | 13 | 48 | 14 | 0 | 0 | 3 |
| KO40 | 400 | 13 | 36 | 14 | 0 | 1 | 3 |
| 40BP6 | 400 | 4 | 38 | 5 | 0 | 0 | 1 |

[a] Abnormal cells excluding gaps.
[b] Ctd: chromatid.

Table 2
Chromosomal aberrations in wild-type and *fancg* cells treated with MMS[a]

| Cell line | MMS dose (μg/ml) | Cells scored | Abnormal cells[b] | Ctd[c] gaps | Ctd breaks | Ctd exchanges | Other | Percentage abnormal cells |
|---|---|---|---|---|---|---|---|---|
| AA8 | 0 | 150 | 2 | 21 | 2 | 0 | 0 | 1.3 |
| AA8 | 200 | 150 | 9 | 26 | 8 | 1 | 0 | 6.0 |
| KO40 | 0 | 200 | 3 | 18 | 3 | 0 | 1 | 1.5 |
| KO40 | 200 | 150 | 7 | 24 | 5 | 1 | 0 | 4.7 |

[a] Cells were examined at the 14 h time point in one of the two cell cycle experiments shown in Fig. 4.
[b] Abnormal cells excluding gaps.
[c] Ctd: chromatid.

mutagenesis that presumably induced numerous adventitious mutations [51,52]. Our study confirms and extends previous findings on those mutants with regard to their sensitivity to a broad-spectrum of DNA damages by providing quantitatively robust data [32,35]. Several phenotypes of these earlier mutants differ from KO40 cells. For example, UV40 and NM3 were reported to have substantial IR sensitivity (two- to three-fold), but KO40 is only slightly sensitive (∼1.2-fold). The elevated SCE seen in UV40 [52] is not present in KO40 (or in NM3 [32]). Importantly, the morphology and slow growth rate of both UV40 and NM3 differ markedly from AA8, which has the same morphology as KO40. However, the sensitivity of NM3 cells to the DNA strand-breaking agents bleomycin and streptonigrin [32] is also present in KO40 cells. The phenotype of KO40 cells is subtle as its growth properties are almost normal in the absence of genotoxic stress. Knockout of *FancG* in the mouse resulted in nearly normal animals without major developmental defects, anemia, or elevated cancers [53,54]. At the cellular level, mouse *fancg* mutant cells are similar to human FA-G cells.

### 4.2. Fancg cells do not have a recombination-defective phenotype

The broad genotoxicity profile of *fancg* cells provides insight into FancG function with respect to the hypothesis that the FA "pathway" may act primarily in HRR [2]. KO40 cells differ significantly in their profile of mutagen sensitivity from both human FA cells and from hamster cell mutants that are defective in HRR. The following four findings argue against an HRR defect in *fancg* cells. First, their sensitivity to crosslinking agents is much lower (three–four-fold) than that of the *xrcc2* and *xrcc3* hamster cell mutants (60–100-fold), which are defective in HRR [55–57]. Second, again in contrast to *xrcc2* and *xrcc3* mutants, *fancg* cells have only slight IR sensitivity [56,58–60]. Third, if the defect in *fancg* cells were in the HRR machinery per se, we would expect to see greatly increased spontaneous aberrations [56,60,61] and reduced SCEs, but we do not. Fourth, in S-phase cells both the spontaneous and MMS-induced levels of Rad51 foci are *increased*, suggesting that HRR activity is elevated rather than reduced. All these differences indicate that FancG is not needed for efficient HRR.

The phenotype of a recent *fancg* mutant of chicken DT40 cells [62] is somewhat similar to KO40. The DT40 mutant has no IR sensitivity to killing in an asynchronous culture but shows some sensitivity to IR-induced chromosomal aberrations in G2 phase, a feature that we did not find (data not shown). The DT40 mutant is sensitive to killing by cisplatin and (slightly) MMC, and also has reduced gene targeting and markedly reduced homologous recombination mediated by a DSB produced in an I-*Sce*I substrate. Although these latter results might suggest a general defect in homologous recombination repair, the substrates in these assays are not physiological and may not mimic events occurring during DNA replication. Like KO40, the DT40 *fancg* mutant is HRR-competent as measured by SCE, Rad51 foci, and spontaneous chromosomal aberrations.

### 4.3. The sensitivity of fancg cells to methylation damage and 6-thioguanine may result from defective processing of single-strand breaks or gaps by the DNA replication machinery

The two- to four-fold sensitivity of KO40 cells to methylating and ethylating agents is worthy noting. This property

is generally not seen with FA lymphoblasts. However, line AA8, like other CHO cells, lacks detectable $O^6$-meG methyltransferase activity, which likely contributes to their hypersensitivity to alkylation damage [63,64]. Thus, when DNA replication encounters a high burden of $O^6$-meG from MNU exposure, it will result in $O^6$-meG:T base pairs, which are a substrate for mismatch repair. Mismatch excision may result in gaps that will persist through a futile loop of excision and resynthesis [65]. In the next S phase, a gap will be subject to conversion to a potentially lethal DSB in one daughter chromatid. We propose that FancG helps the replication machinery proceed across such a gap and prevent an overt DSB from arising (loss of chromatid continuity). Similarly, the sensitivity of *fancg* cells to methylation by MMS and MNU, which produce different proportions of $O^6$-meG [66], suggests that single-strand breaks arising during base excision repair may be converted during DNA replication to DSBs more readily in *fancg* cells than in wild type.

The high sensitivity of KO40 cells to $S^6$G is a new, intriguing finding for an *FA* gene mutation. The toxicity to KO40 from $S^6$G occurs at ∼2% substitution, a lesion density that is orders of magnitude higher than for crosslinks. Incorporated $S^6$G becomes methylated to form $S^6$-me-$S^6$G, which upon replication directs the incorporation of either T or C. $S^6$-me-$S^6$G:T pairs are recognized by the mismatch repair system [67,68]. Thus, the increased single-strand breaks and defective elongation of the nascent strand in $S^6$G-containing DNA [69] may result from mismatch-repair incisions. A reduced ability to coordinate DNA replication with a high rate of mismatch repair, resulting in excessive DSBs arising at replication forks, may explain the sensitivity of *fancg* cells to $S^6$G. Future studies will be needed to determine whether there is any clear link between FA proteins and the mismatch repair machinery.

### 4.4. FancG may serve to promote translesion synthesis at replication-blocking lesions

Various other defects have been proposed to underlie FA. A defect in non-homologous end joining in FA cells, and also the UV40 *fancg* mutant, was proposed, partly based on increased sensitivity to killing by a transfected restriction enzyme, *Pvu*II [70]. However, we saw no differential sensitivity between KO40 and AA8 cells in such experiments (unpublished results). Greatly increased Rad51 protein level was also reported for nonimmortalized FA fibroblasts, including FA-G [71], but we saw no change in Rad51 level in either KO40 cells (reported here) or EUFA143 lymphoblasts compared with parental or gene-complemented controls (unpublished results).

In summary, our data indicate that FancG in CHO cells helps minimize the potential lethality associated with a variety of DNA lesions including base methylation, bulky monoadducts, crosslinks, and UV-C photoproducts. As *fancg* mutant cells differ markedly in phenotype from HRR-defective mutants such as *xrcc2* and *xrcc3*, we infer that FancG acts during DNA replication [72], separately from HRR. The broad mutagen sensitivity profile of *fancg* CHO cells suggests a more global role for the FA proteins in maintaining chromatid continuity when the replication machinery encounters nicks, gaps, and blocking lesions. An important clue as to the central role of FA proteins may be the observation that FA cells were shown to have greatly reduced *hprt* mutability for both monofunctional and bifunctional psoralens [73,74]. In normal cells the majority of spontaneous and psoralen-induced *hprt* mutants are point mutations whereas deletion mutations predominate in FA cells [73]. In FA cells, the *$Na^+/K^+$-ATPase* locus is also hypomutable by photoaddition of bifunctional psoralens [74]. These observations suggest that the FA proteins might promote translesion synthesis by error-prone polymerases such as Rev1 and Rev3 [75,76]. In the absence of translesion synthesis at blocking lesions, DNA replication forks may be prone to collapse, causing deletion mutations, viable chromosomal rearrangements, and cell lethality.

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